

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re US Patent Application No. 10/552,000

For: METHOD OF PRODUCING CHONDROCYTES FOR TRANSPLANTATION

VERIFICATION OF TRANSLATION

Honorable Commissioner of Patents and Trademarks

Washington, D.C. 20231

Sir :

I, Katsufumi HIRATA, residing at 20-25, Nishishinagawa 3-chome, Shinagawa-ku, Tokyo, Japan, declare and state :

- (1) that I am able to read and understand well both the Japanese and English languages;
- (2) that I supervised the translation of the Japanese Patent Application No. 176351/2003 from Japanese to English;
- (3) that the attached English translation is a true and correct translation of the above-identified International Application to the best of my knowledge and belief; and
- (4) that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001, and that such false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: May 22, 2006

Katsufumi Hirata  
Signature: Katsufumi HIRATA

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【Name of Document】 SPECIFICATION

【Name of the Invention】 METHOD OF PRODUCING CHONDROCYTES FOR  
TRANSPLANTATION

【Claims】

【 Claim 1 】 A method of producing human chondrocytes characterized by comprising co-culturing chondrocytes obtained from a cartilage having perichondrium together with the perichondrium.

【Claim 2】 The production method as claimed in claim 1 characterized in that the cartilage is auricular cartilage.

【Claim 3】 The production method as claimed in claim 1 characterized by comprising monolayer or multilayer seeding the cultured cells once or more and culturing to give a chondrocyte mass.

【Claim 4】 The normal human chondrocytes produced by the method as claimed in claim 1 to 3.

【Claim 5】 A cartilage therapy material comprising human chondrocytes obtained by a method as claimed in claim 4 either alone or together with an embedding material.

【Claim 6】 The cartilage therapy material as claimed in claim 5 wherein the embedding material is one or more members selected from among collagen, polyglycolic acid, polylactic acid, alginic acid, polyethylene oxide, fibrin adhesive, polylactic acid-polyglycolic acid copolymer, proteoglycan, glucosaminoglycan or human dermis.

**【Detailed Description of the Invention】**

**【0001】**

**【Technical Field to which the Invention Pertains】**

This invention relates to a method of producing normal human chondrocytes and the normal human chondrocytes obtained by the method. It also relates to a cartilage therapy material using the thus obtained normal human chondrocytes.

**【0002】**

**【Prior Art】**

In a cartilage tissue, chondrocytes exist in the state of being embedded in the matrix. These chondrocytes can be separated from the matrix by treating the cartilage with an enzyme such as collagenase. Attempts have been made to utilize these separated chondrocytes in transplantation therapy, in particular, autotransplantation of chondrocytes for treating cartilage-related diseases. It has been experimentally confirmed that transplantation therapy with the use of this method is applicable to animals such as rabbits and cows from which a large amount of cells can be obtained (see, for example, Bentry, et al., Nature 230: 385-388 (1971), Green, Clin. Orthop. 124:237-250 (1977); Wakitani et al., J. Bone and Joint Surgery 71B: 74-80 (1989); Paige et al., Plastic and Reconstructive Surgery 96:1390-1398 (1995); and Paige et al., Plastic and Reconstructive Surgery 97:168-178 (1996)).

**【0003】**

Attempts have been also made to culture human chondrocytes in, for example, articular cartilage, auricular cartilage and costal cartilage (Aulthouse et al., In Vitro Cellular & Developmental Biology 25: 659-668(1989); Brittberg et al., The New England Journal of Medicine 331: 889-895(1994); Ting et al., Annals of Plastic Surgery 40: 413-421(1998); and Rodriguez et al., Plastic and Reconstructive Surgery 103:1111-1119(1999)).

**【0004】**

In humans, however, only a small amount of cartilage can be collected, therefore, only a small number of chondrocytes can be used at the initiation of the culture. Moreover, human chondrocytes can be minimally proliferated by the conventional methods and proliferated chondrocytes, if any are obtained, convert into fibroblasts having different characters. Thus, it is highly difficult to apply human chondrocytes to transplantation therapy in practice. Namely, there is a problem that although a large amount of normal chondrocytes is required for transplantation in humans, it has been impossible to obtain a sufficient amount of human chondrocytes using the currently available methods.

**【0005】**

To overcome this problem, the present inventor proposed to quickly culture a large amount of human chondrocytes by co-culturing human chondrocytes together with perichondral

cells in the chondrogenic stage serving as feeder cells supporting the proliferation ability of the chondrocytes (WO 02/12451 (2002)). The use of nonhuman animal feeder cells is, however, accompanied with problems of unexpected bacterial or viral infections and complicated treatments are needed to prevent these infections.

**【0006】**

**【Non Patent Document 1】**

Bentry, et al., Nature 230: 385-388(1971)

**【Non Patent Document 2】**

Green, Clin. Orthop. 124:237-250(1977)

**【Non Patent Document 3】**

Wakitani et al., J. Bone and Joint Surgery 71B: 74-80  
(1989)

**【Non Patent Document 4】**

Paige et al., Plastic and Reconstructive Surgery  
96:1390-1398(1995)

**【Non Patent Document 5】**

Paige et al., Plastic and Reconstructive Surgery  
97:168-178(1996)

**【Non Patent Document 6】**

Aulthouse et al., In Vitro Cellular & Developmental  
Biology 25: 659-668(1989)

**【Non Patent Document 7】**

Brrittberg et al., The New England Journal of Medicine

331: 889-895(1994)

【Non Patent Document 8】

Ting et al., Annals of Plastic Surgery 40: 413-421(1998)

【Non Patent Document 9】

Rodriguez et al., Plastic and Reconstructive Surgery  
103:1111-1119(1999)

【0007】

【Patent Document 1】

WO 02/12451 (2002)

【0008】

【Problem to be solved by the Invention】

An object of the present invention is to provide a method to quickly obtain a large amount of normal human chondrocytes or a mass thereof without fear of bacterial or viral infection. Another object of the present invention is to provide a cartilage therapy material with the use of the normal human chondrocytes or a mass thereof thus obtained.

【0009】

【Means for Solving Problem】

The present invention relates to a method of producing human chondrocytes characterized by comprising co-culturing chondrocytes obtained from a cartilage having perichondrium together with the perichondrium, especially a method of producing human auricular cartilage. And the present invention relates to the product i.e., normal human chondrocytes, in

particularly, human auricular cartilage produced by said method.

**【0010】**

The present invention is a production method as described in the above characterized by comprising monolayer or multilayer seeding of the cultured cells once or more and culturing to give a chondrocyte mass, in particularly human auricular cartilage mass.

**【0011】**

Further the present invention relates to a cartilage therapy material comprising normal human chondrocytes obtained by said method as described above either alone or together with an embedding material wherein the embedding material is one or more members selected from among collagen, polyglycolic acid, polylactic acid, alginic acid, polyethylene oxide, fibrin adhesive, polylactic acid-polyglycolic acid copolymer, proteoglycan, glucosaminoglycan and human dermis.

**【0012】**

**【Mode for Carrying out the Invention】**

By further improving the preliminarily proposed method with the use of feeder cells (WO 02/12451 (2002)), the present inventor has established a more convenient method of culturing and proliferating chondrocytes such as auricular cartilage cells without resorting to feeder cells. Although it has been considered that feeder cells are required in culturing and



proliferating chondrocytes, the present inventor has found out that chondrocytes can be cultured and proliferated without resorting to feeder cells. That is to say, the method according to the present invention can be conveniently carried out while omitting the troublesome procedures needed in the case of using feeder cells and, moreover, the risk of infection from the feeder cells and so on can be avoided thereby. Thus, it can be said that this method is highly safe and most suitable for autotransplantation in humans.

**【0013】**

It has been further found out that a cartilage mass, which is obtained by multilayer culturing human chondrocytes that have been proliferated by the above-described method, is usable in transplantation therapy without resorting to a support.

**【0014】**

In the case where the human cartilage to be used in the present invention is auricular cartilage, it is favorable from a cosmetic viewpoint to make a slight incision in the post auricular matrix skin and collect a cartilage tissue of a small size (about 1x1 cm<sup>2</sup>). It is favorable that the thus collected cartilage tissue has perichondrium bonded to one face. Owing to this procedure, the cartilage can be quickly regenerated from the perichondrium remaining on the other face at the cartilage collection site and, as a result, the overall healing can be rapidly completed.

【0015】

To obtain cells required for transplantation, the auricular cartilage having the perichondrium thus collected is finely diced and subjected to monolayer culture. In this step, it is preferable to employ a culture medium containing a cytokine required in the proliferation of the chondrocytes. Transplantation can be performed merely by using the cells obtained by this monolayer culture. By further subjecting the cells thus proliferated by the monolayer culture to multilayer culture several times, it is possible to obtain a tissue that has a high mechanical strength and therefore is durable when handled with instruments such as tweezers and never undergoes dispersion or absorption *in vivo* after transplantation. Although the number of multilayer seeding varies depending on the size of a desired tissue, it is generally preferable to carry out the multilayer seeding three or four times.

【0016】

The thus obtained tissue is fed into an injection syringe or the like and, after attaching a needle, is then injected into a defect in a cartilage to thereby treat or repair nasal deformation, nose elevation, facial bone deformation, facial bone defect, gnathoplasty, skull deformation, skull defect, arthrosis deformans, microtia or other diseases accompanied by a defect in a cartilage or a defective cartilage. In this step, the cartilage tissue may be used in the form of a mixture with

a carrier selected from among collagen, polyglycolic acid (PGA), polylactic acid, alginate, polyethylene oxide, fibrin adhesive, polylactic acid-polyglycolic acid copolymer, proteoglycan or glucosaminoglycan. The chondrocytes obtained by the production method according to the present invention are practically usable as such without resorting to a carrier.

**【0017】**

Human chondrocytes

The method of producing chondrocytes according to the present invention can be applied to the culture of chondrocytes of any human cartilage tissue having perichondrium bonded thereto such as auricular cartilage, costal cartilage, articular cartilage, intervertebral cartilage or tracheal cartilage. In particular, it is suitable for culturing and proliferating chondrocytes of auricular cartilage.

**【0018】**

The chondrocytes to be used in the production method according to the present invention can be obtained from a human cartilage tissue having perichondrium by publicly known methods. It is generally preferable that an excised cartilage tissue is diced with a surgical knife or the like, treated with collagenase and then cultured and proliferated.

(1) A cartilage tissue is excised and disinfected by allowing to stand at about 4°C overnight together with an antibiotic (for example, penicillin or kanamycin) or an

antifungal agent (for example, amphotericin B). Then, the cartilage tissue is diced with a scalpel, etc.

(2) The diced cartilage tissue is then transferred into a medium containing type II collagenase and allowed to stand at about 4°C overnight. Next, it is shaken at 37°C for 4 hours.

(3) The thus treated tissue is centrifuged and the obtained precipitate is employed in the culture.

**【0019】**

By this method, 3 to  $5 \times 10^6$  chondrocytes can be obtained at the first generation of subculture from a human auricular cartilage tissue piece (1 cm<sup>2</sup>). In the culture method according to the present invention, known growth factors can be used, especially ones capable of stimulating the proliferation of cartilage. Appropriately selected forms among FGF (for example, bFGF), IGF (for example, IGF-I), bone morphogenetic protein 9 (BMP-9) or combinations thereof.

**【0020】**

Method of culturing human chondrocytes

To culture human chondrocytes, use can be made of publicly known media suitable for culturing chondrocytes. In addition to fetal bovine serum (FBS) or human serum and hydrocortisone, the media may optionally contain a proliferation factor such as human bFGF or human IGF-I (Cuevas et. al., Biochem. Biophys. Res. Commun. 156, 611-618 (1988); and Froger-Gaillard et al., Endocrinol. 124, 2365-72). As an example of such a medium,

DME(H) medium containing FBS (preferably about 10%), human bFGF (preferably about 10 ng/ml), hydrocortisone (preferably 40 ng/ml) and human IGF-I (preferably 5 ng/ml) can be cited. It is also possible to use autoserum obtained from a patient himself/herself so as to enhance the safety.

**【0021】**

1) Primary Culture

The chondrocytes are seeded in a medium contained in a flask and cultured in a CO<sub>2</sub> incubator under such conditions as being suitable for the culture of chondrocytes (for example, at 37°C and 10% CO<sub>2</sub>). The culture is continued until the proliferated cells form a confluent monolayer (usually for 10 to 14 days).

2) Subculture

The subculture can be carried out with the use of the same culture medium as the primary culture (usually 7 days per subculture). In the case of subculturing auricular cartilage-origin cells obtained by the primary culture, the cell count increases about 1000 times from P0 (primary culture) to P4. When a larger number of chondrocytes are needed, the subculture can be further repeated.

3) Multilayer Culture

A gel-like chondrocyte mass can be obtained by multilayer seeding and culturing the subcultured human chondrocytes once or more, preferably 3 to 4 times. In the chondrocyte mass thus

obtained, the human chondrocytes are surrounded by a cartilage matrix containing aggrecan, etc. and the cells are bonded to each other via the matrix such as aggrecan to form a gel-like cell mass.

**【0022】**

**Cartilage Therapy Material**

The above described sub cultured or multilayer cultured human chondrocytes or the cell mass obtained by the present invention, either as such or in the state of being embedded in a biomaterial, can be used in transplantation as a cartilage therapy material. Examples of the carrier in which the human chondrocytes or the cell mass can be embedded include collagen, polyglycolic acid (PGA), polylactic acid, alginate, polyethylene oxide, fibrin adhesive, polylactic acid-polyglycolic acid copolymer, proteoglycan, glycosaminoglycan and mixtures thereof. Besides that mentioned above, there may be a preferable case in which the carrier for embedding chondrocytes or the cell mass is not used because of that absorption of chondrocytes followed by absorbing of the carrier does not occur.

**【0023】**

By appropriately selecting and combining the carriers in which the chondrocytes are embedded, the cartilage therapy material can induce not only chondrogenesis but also cartilaginous ossification. As an example of the carrier

enabling the induction of the cartilaginous ossification, human dermis may be cited. Moreover, it is possible to further promote the ossification by using a growth factor capable of promoting osteogenesis such as a bone morphogenetic protein (BMP).

**【0024】**

To further illustrate the present invention in greater detail, but not by way of restriction, the following examples will be given.

**【0025】**

Example 1: Chondrocyte culture

Composition of culture medium: To the DME(H) medium, 10% of FBS, 10 ng/ml of human FGF (Kaken Pharmaceutical Research Institute), 40 ng/ml hydrocortisone (Sigma) and 5 ng/ml human IGF-I (GIBCO) were added. Collected cartilage: From human rear auricular cartilage, a sample piece (about 1x1 cm<sup>2</sup>) having perichondrium bonded to one face was collected.

**【0026】**

(a) Chondrocyte fraction

The cartilage piece obtained above was disinfected with penicillin G (800 u/ml), kanamycin (1 mg/ml) and Fungizone (2.5 ug/ml). It was then diced with a scalpel and then allowed to stand in F-12 medium containing 0.3% of type II collagenase (Worthington Biochemical) at 4°C overnight. On the next day, the culture medium was shaken at 37°C for 4 hours and centrifuged.

The precipitate thus obtained was employed as a cell fraction at the initiation of the culture.

【0027】

(b) Primary Culture

The cell fraction as described above was seeded by using the above-described medium in a flask having a base area of 75 cm<sup>2</sup>. The cell fraction in this flask was cultured in a CO<sub>2</sub> incubator at a CO<sub>2</sub> concentration adjusted to 10%. The culture medium was replaced twice a week. As a result, the chondrocytes formed a confluent monolayer within a culture time of 10 to 14 days. The obtained cells were used for the following subculture.

【0028】

It was confirmed that similar results were obtained by using, as the substitute for FBS, autoserum obtained by centrifuging the autoserum of a patient at 3000 rpm for 10 minutes.

【0029】

(c) Subculture

Subculture was carried out by seeding  $1 \times 10^6$  of the primary-cultured cells in a flask having a base area of 175 cm<sup>2</sup> and employing the same conditions as the primary culture. After culturing for 7 days, the cells formed a confluent monolayer. The obtained cells were employed in the next subculture. As a result, the cell count on the fourth



subculture increased about 1000 times, compared with the cell count at the initiation of the subculture.

**【0030】**

(d) Multilayer Culturing

The chondrocytes obtained by the subculture were seeded by overlaying thrice at a density of  $1 \times 10^6$  cells/cm<sup>2</sup> and cultured by multilayer culturing. After culturing for 2 weeks, a sheet-like gel mass was formed (FIG. 1). When the gel-like chondrocyte mass was stained with hematoxylin-eosine (HE), it was observed that the cells were multilayered and bonded together via the matrix (FIG. 2). When the cells were immunologically stained for type II collagen serving as a molecular marker of cartilage tissue, the extracellular matrix was stained, thus indicating that the extracellular matrix was a cartilage-specific matrix (FIG. 3).

**【0031】**

Example 2: Transplantation of chondrocytes

First, the medium was removed from the culture flask and the cells were harvested with a cell lifter. Then the harvested cells were collected with a syringe. Next, the chondrocytes collected into the syringe were injected into a defect in a cartilage of a nude mouse by using an injection needle. Six months after the transplantation, a specimen was sampled from the transplantation site and histologically examined to confirm the success in engraftment. When the sampled tissue was stained

with hematoxylin-eosine (HE), namely, it was observed that cells were multilayered and bonded to each other via the matrix (FIG. 4). When the cells were immunologically stained for type II collagen serving as a molecular marker of cartilage tissue, the extracellular matrix was stained, indicating that the extracellular matrix was a cartilage-specific matrix (FIG. 5). Moreover, metachromasia was shown in toluidine blue staining, indicating the presence of aggrecan serving as a cartilage marker (FIG. 6). These results indicated that the transplanted chondrocytes had formed a cartilage tissue.

【0032】

【Effect of the Invention】

According to the present invention, a large amount of normal human chondrocytes or a mass thereof can be quickly obtained in a large amount without fear of bacterial or viral infection.

【Brief Description of Drawings】 Brief Description of Drawings

Fig. 1 presents a photograph showing a sheet-like gel mass obtained after multilayer culturing chondrocytes have been sub cultured for 2 weeks.

Fig. 2 presents a photograph showing the result of hematoxylin-eosine staining of the gel-like chondrocyte mass shown in Fig. 1.

Fig. 3 presents a photograph showing the result of immunological staining for type II collagen serving as a

cartilage tissue molecular marker.

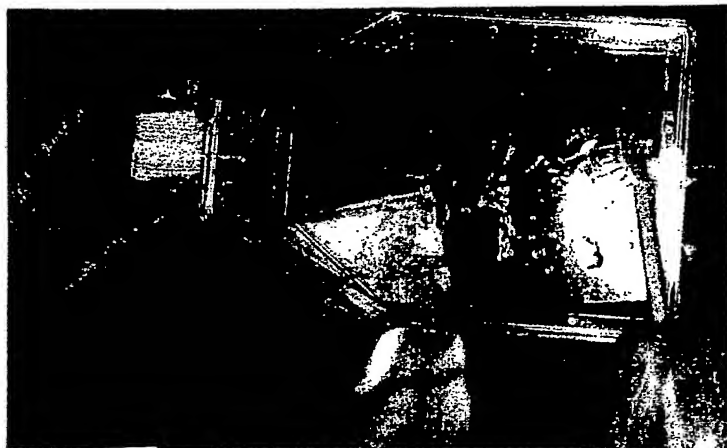
Fig. 4 presents a photograph showing the result of hematoxylin-eosine (HE) staining of a specimen sampled from a transplantation site.

Fig. 5 presents a photograph showing the result of immunological staining of a specimen sampled from a transplantation site for type II collagen serving as a cartilage tissue molecular marker.

Fig. 6 presents a photograph showing the result of toluidine blue staining of a specimen sampled from a transplantation site.

**[Name of Document] DRAWINGS**

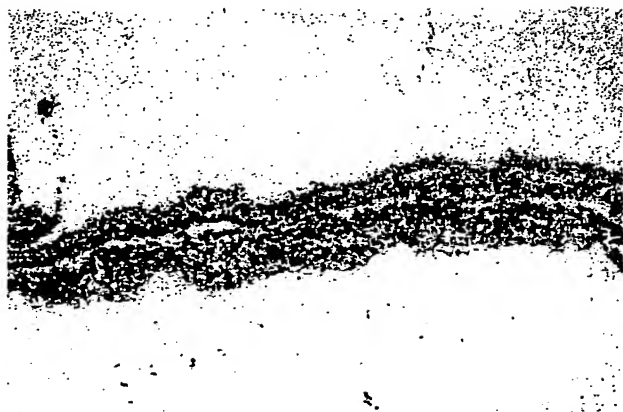
**[Fig.1]**



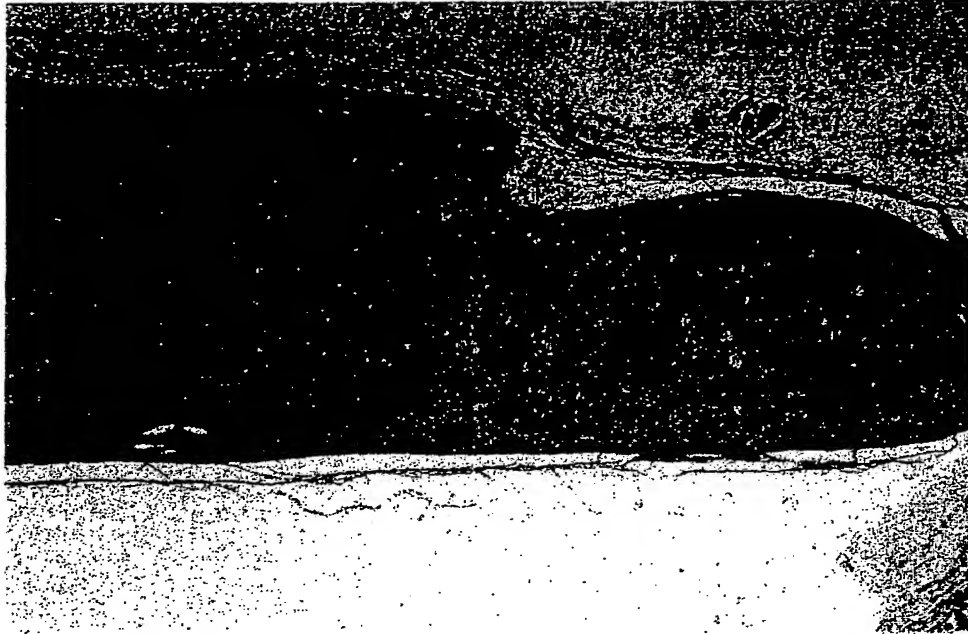
**[Fig.2]**



**[Fig.3]**



**[Fig.4]**



**[Fig.5]**



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**【Fig.6】**



【Name of Document】 ABSTRACT

【Abstract】

【Problem】

This invention is intended to provide a method to quickly obtain a large amount of normal human chondrocytes or a mass thereof without fear of bacterial or viral infection.

【Means for solving the Problem 】

This invention is a method of producing human chondrocytes characterized by comprising co-culturing chondrocytes obtained from a cartilage having perichondrium, for example, auricular cartilage together with the perichondrium; and a method of producing human chondrocytes characterized by comprising monolayer or multilayer seeding the cultured cells once or more and culturing to give a chondrocyte mass.

【Selected Drawings】 None